JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2009, 60, 4, 93-99
www.jpp.krakow.pl

F.E. MARTIN-CANO, P.J. GOMEZ-PINILLA, M.J. POZO, P.J. CAMELLO

SPONTANEOUS CALCIUM OSCILLATIONS IN URINARY BLADDER SMOOTH MUSCLE CELLS

Department of Physiology, Faculty of Veterinary Sciences and Nursing School, University of Extremadura, 10071 Caceres, Spain

Although spontaneous phasic activity of detrusor muscle plays an important role in urinary bladder function there is little information regarding myogenic $[Ca^{2+}]_i$ signals in this tissue. We have studied spontaneous, unstimulated $[Ca^{2+}]_i$ signals in fura-2 loaded detrusor cells isolated from newborn (10-13 days old) guinea-pig urinary bladder. In newborn guinea pigs 35% of studied muscle cells displayed spontaneous $[Ca^{2+}]_i$ oscillations with several kinetic patterns (from irregular to highly paced cycles). The oscillations were inhibited by external Ca^{2+} removal, treatment with L- and T-type Ca^{2+} channel blockers and by the hyperpolarizing drug pinacidil. Ca^{2+} stores were necessary to maintain oscillations, as indicated by the inhibitory effects of thapsigargin, ryanodine and 2-APB. Oscillations were also inhibited by folimycin, an inhibitor of acidic Ca^{2+} stores. Treatment with the selective inhibitors iberiotoxin and NPPB indicated that the oscillatory signal is also modulated by Ca^{2+} -activated K^+ channels (inhibitory) and Ca^{2+} -activated Cl^- channels (stimulatory). Our results indicate that detrusor cells from newborn guinea-pigs develop spontaneous $[Ca^{2+}]_i$ oscillations due to Ca^{2+} influx through T- and L-type Ca^{2+} channels modulated by intracellular stores, including acidic pools. This activity could underlie the myogenic activity of urinary bladder during early stages of development.

Key words: *Ca^{2+} stores, detrusor, Ca^{2+} channels*

INTRODUCTION

Urinary bladder smooth muscle is a spontaneously active tissue, showing phasic contractions associated to different patterns of spontaneous action potentials and $[Ca^{2+}]_i$ transients (1-3). To accommodate urine during the filling phase of micturition, the electrical coupling between bundles of myocytes is limited, avoiding the spread of emptying contractions while allowing the phasic activity to adjust the length of individual bundles to an optimal (minimal surface area). During micturition, detrusor muscle contracts in response to parasympathetic fibers. The phasic activity of bladder muscle is increased in the perinatal period (4), but there is no information about the underlying mechanisms for this increase.

The available information regarding this activity comes from studies using strips or bundles of muscle cells, where $[Ca^{2+}]_i$ signals from single cells have not been discerned until a recent report in mouse bladder strips (2). On the contrary, spontaneous activity has not been reported in isolated bladder muscle cells, a model used to characterize several elements of $[Ca^{2+}]_i$ homeostasis (5-7) and plasma membrane excitation mechanisms (8).

Rhythmic smooth muscle activity is considered to be due to interstitial cells of Cajal (ICC), extensively studied in the gastrointestinal tract but also present in urinary bladder (9). Previous reports also points to ICC cells as the origin for bladder spontaneous Ca^{2+} oscillations, although in bundles of detrusor muscle spontaneous action potentials and the subsequent $[Ca^{2+}]_i$ signals are initiated in non typical ICC-like cells situated at the border of the bundles (1, 10).

The ultimate origin of the activity is unknown, but cross-talk between Ca^{2+}-activated K^+ channels (BK and SK), voltage-operated Ca^{2+} channels and intracellular Ca^{2+} stores has been reported to be involved in phasic contractions of detrusor strips (11). In bundles of detrusor muscle the $[Ca^{2+}]_i$ oscillations have been attributed to Ca^{2+} influx through voltage-operated channels, which initiate the action potentials and the subsequent $[Ca^{2+}]_i$ transients (12). The role of intracellular Ca^{2+} stores would be to amplify the $[Ca^{2+}]_i$ increase induced by the Ca^{2+} influx through Ca^{2+} induced Ca^{2+} release (CICR), but is not essential for the maintenance of phasic activity (10, 13).

The aim of our study was to characterize spontaneous Ca^{2+} signals in guinea-pig isolated detrusor cells and to isolate the homeostatic mechanisms involved. Herein we show that endogenous $[Ca^{2+}]_i$ oscillations are mediated by T- and L-type Ca^{2+} channels and require release from several types of intracellular Ca^{2+} stores, including IP_3 and ryanodine receptors and acidic pools.

MATERIALS AND METHODS

Animals and cell preparation

Detrusor muscle cells were obtained from 10-12 days old guinea-pigs and from 5-6 month old guinea-pigs, after sacrifice following guidelines of University of Extremadura (cervical dislocation after anaesthesia). After removal of urothelium in a sylgard-plated Petri dish filled with Krebs-Henseleit solution (K-

HS, see *Solutions and drugs*), about 20 mg of detrusor was cut into small pieces and incubated (35 min, 37°C) in enzyme solution (ES, see *Solutions and drugs*) supplemented with 1 mg/ml BSA, papain and dithioerythritol. The tissue was then incubated (10 min, 37°C) in fresh ES containing 1 mg/ml BSA and collagenase and 100 µM $CaCl_2$, followed by three washing with ES. Single smooth muscle cells were dispersed with a fire-polished pipette. The resultant cell suspension was kept in ES at 4°C until experiment, within 6 h. Experiments were performed at room temperature.

$[Ca^{2+}]_i$ determination

$[Ca^{2+}]_i$ was determined by epifluorescence microscopy after loading the cells with the ratiometric Ca^{2+} indicator fura 2 (4 µM fura 2-AM, room temperature, 25 min and then stored on ice). Cells were perfused with Na^+-HEPES solution (for composition see *Solutions and drugs*) in a chamber made with a glass poly-D-lysine treated coverslip, on the stage of an inverted microscope (Diaphot TE2000; Nikon). For de-esterification of the dye, >20 min were allowed to elapse before Ca^{2+} measurements. Cells were alternatively excited at 340/380 nm (0.3 Hz) (Optoscan monochromator, Cairn Research). The emitted images, filtered by a 500 nm long-pass filter, were recorded with a cooled CCD camera (ORCAII-ER; Hamamatsu Photonics) and Metafluor 7 (Universal Imaging). After background subtraction, the ratio of 340 and 380 fluorescence (F_{340}/F_{380}) was calculated pixel by pixel and used as index of $[Ca^{2+}]_i$. We did not present data as absolute $[Ca^{2+}]_i$ levels given the uncertainties in establishing the real Kd of fura-2 inside smooth muscle cells, in addition to the difficulty in performing intracellular fura-2 calibration in intact cells due to their rapid deterioration under application of high levels of ionomycin in high- or low-Ca^{2+} solutions.

Solutions and drugs

The K-HS contained (in mM): 113 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11.5 D-glucose. Final pH was 7.35 after gassing with 95% O_2-5% CO_2. The ES used to disperse cells contained (in mM): 10 HEPES, 55 NaCl, 5.6 KCl, 80 sodium glutamate, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The Na^+-HEPES solution contained (in mM): 10 HEPES, 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The Ca^{2+}-free Na^+-HEPES solution was prepared by substituting EGTA (1 mM) for $CaCl_2$. Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: dithioerythritol, HEPES, BSA, bethanechol, thapsigargin, caffeine, 8-Br-cADPr, nitrendipine, folimycin, pinacidil, 2-APB, NPPB and iberiotoxin were from Sigma Chemical (St. Louis, MO); fura 2-AM and ryanodine were from Molecular Probes (Invitrogen, Barcelona, Spain); collagenase was from Fluka (Madrid, Spain); NNC 55-0396 from Tocris and papain was from Worthington Biochemical (Lakewood, NJ). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain).

For inhibitors and activators we chose concentrations ranging from IC_{50}/EC_{50} to those attaining submaximal effects, and previously shown to be efficient and specific for the nominal effect of each compound in contractility studies (see (14) for ryanodine, (15) for nitrendipine, TPS, 2-APB and pinacidil, (16) for folimycin, (17) for NPPB, (18) for NNC and (19) for iberiotoxin).

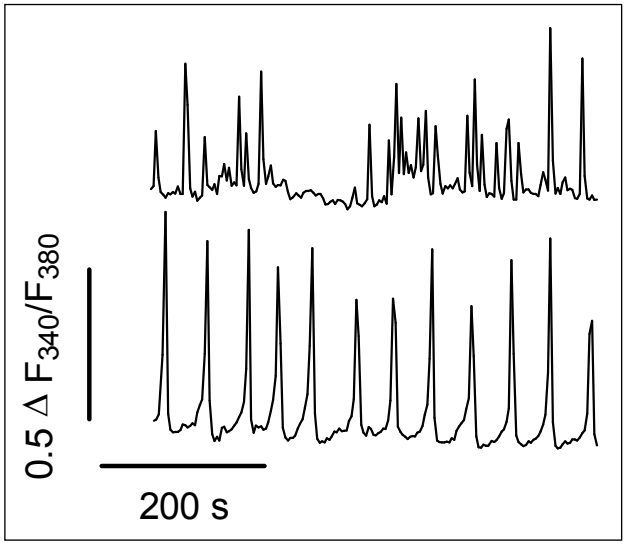

Fig. 1. Smooth muscle cells isolated from urinary bladder of newborn mice show spontaneous $[Ca^{2+}]_i$ oscillations. The traces (raw ratio records of fura-2 -loaded muscle cells) are representative of non stimulated cells developing different patterns of oscillations.

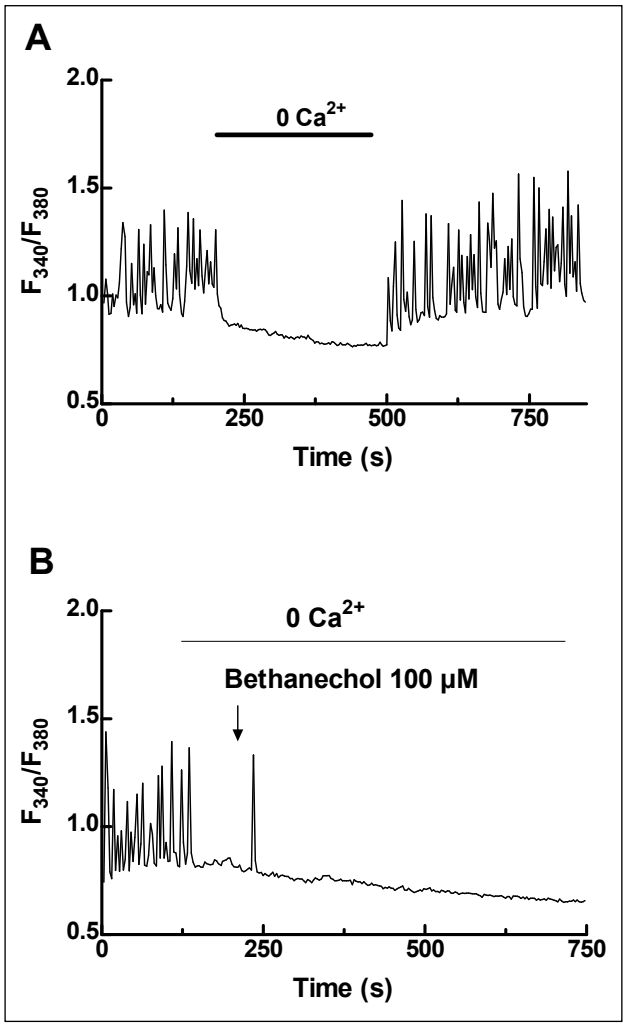

Fig. 2. Spontaneous $[Ca^{2+}]_i$ oscillations of detrusor cells depend upon Ca^{2+} influx. (**A**) Extracellular calcium was transiently removed by replacement of the external solution with a Ca^{2+} -free solution containing 1 mM EGTA. (**B**) After removing extracellular Ca^{2+}, cells were challenged by a maximal concentration of the muscarinic agonist bethanechol, which induced a $[Ca^{2+}]_i$ peak due to release from intracellular stores. Recordings are representative of 19 (**A**) and 13 (**B**) cells from 3 independent preparations.

Data processing and statistics

Frequency (Hz) and amplitude (peak - minimum values of F_{340}/F_{380}) of the $[Ca^{2+}]_i$ oscillations were analyzed using Mini Analysis (Synaptosoft, USA) or Chart5 Pro (ADInstruments, USA). Mean values are presented as average ± S.E.M. (standard error of the mean). Statistical differences between two means were determined by Student's t-test, and considered significant if $P<0.05$ or lower. All the experiments were performed at least in three different preparations.

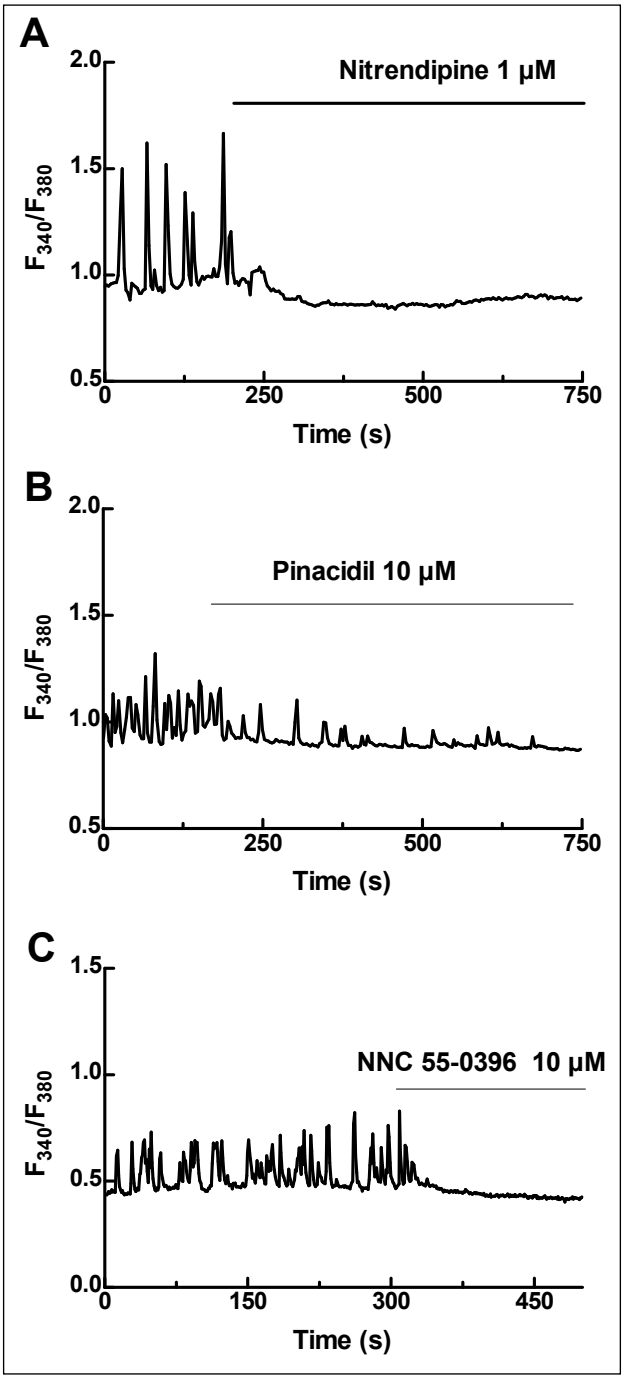

Fig. 3. Voltage-activated Ca^{2+} channels are responsible for spontaneous Ca^{2+} oscillations. (**A**) Treatment of cells with nitrendipine, a specific blocker of L-type calcium channels, inhibited the oscillatory signal. (**B**) Inhibitory effect of pinacidil, a K_{ATP} channel opener, on the oscillations of detrusor smooth cells. (**C**) Application of the specific blocker of T-type Ca^{2+} channel NNC 055-0396 suppressed the oscillations. Traces are typical of 8 (A), 17 (B) and 6 (C) cells, from at least 3 independent preparations.

RESULTS

In our experimental conditions, the percentage of cells showing $[Ca^{2+}]_i$ oscillations was very low in adult animals (less than 5% of the microscope fields). In the case of cells isolated from newborn mice 50% of microscope fields contained oscillatory cells. Overall, a 35.4% of the recorded smooth muscle cells displayed spontaneous $[Ca^{2+}]_i$ oscillations, with a mean amplitude of 0.252±0.009 $\Delta F_{340}/F_{380}$ and an average frequency of 0.037±0.001 Hz (185 cells). The low percentage of oscillating cells found in adult smooth muscle made it impractical to characterize the oscillations in adult mice.

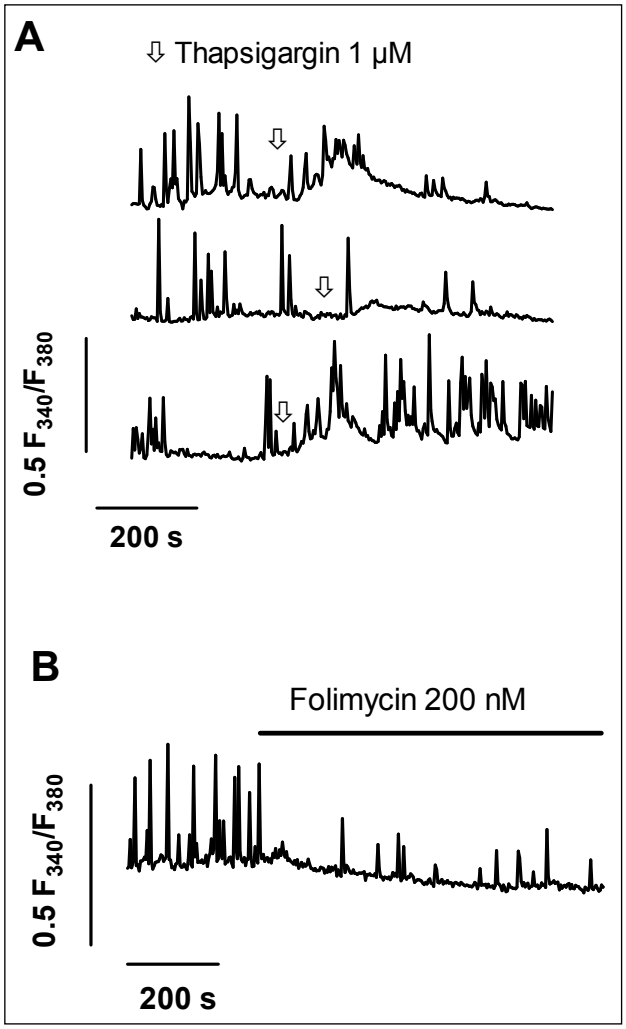

Fig. 4. Inhibition of SERCA Ca^{2+} pump or V-type proton pump of the stores modifies $[Ca^{2+}]_i$ oscillations. (**A**) Typical responses of oscillating detrusor cells to application of the specific SERCA inhibitor thapsigargin (arrow, TPS). More than 70% of the cells showed a delayed (top) or fast (middle) inhibition of the oscillations. In the remaining 27% of cells TPS increased the frequency in the time-frame of the record. Records are typical of 15-22 cells from 5-7 different preparations. (**B**) Effect of folimycin, a specific inhibitor of V-type proton pumps, on Ca^{2+} oscillations. Representative of 36 cells from 5 preparations.

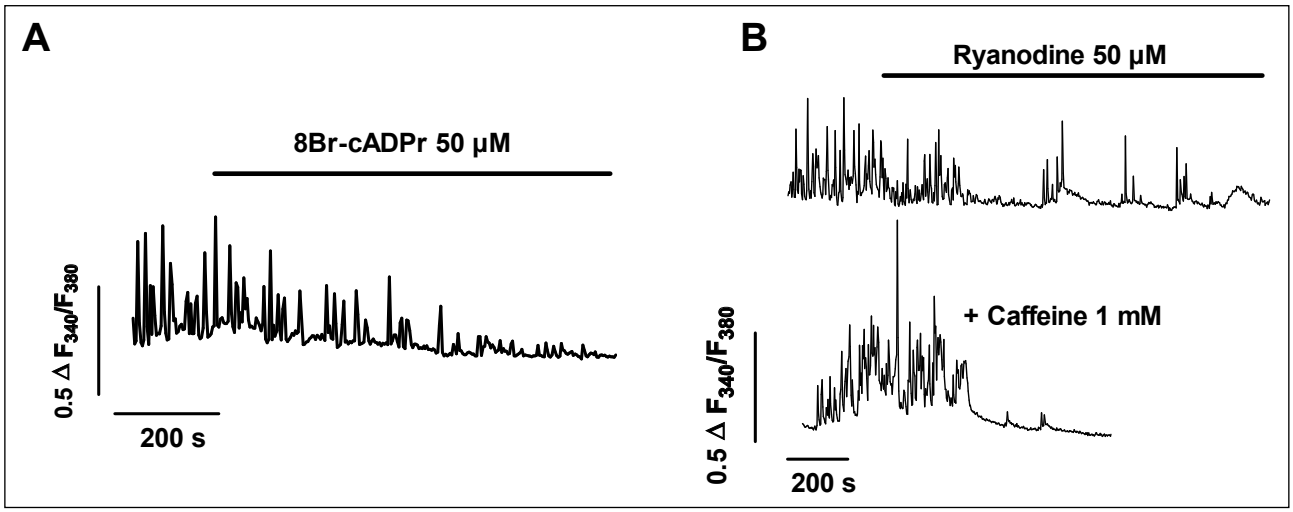

Fig. 5. $[Ca^{2+}]_i$ oscillations are inhibited by antagonists of ryanodine receptors. (**A**) Application of the antagonist of ryanodine receptor, 8-Br-cADPr, inhibited spontaneous oscillations in mice detrusor cells. Typical of 15 cells. (**B**) Inhibitory effect of ryanodine (top). Co-application with 1 mM caffeine (bottom trace) induced a faster inhibition of the oscillations. Traces are representative of 27 (top) and 8 (bottom) cells from 6 and 4 preparations respectively.

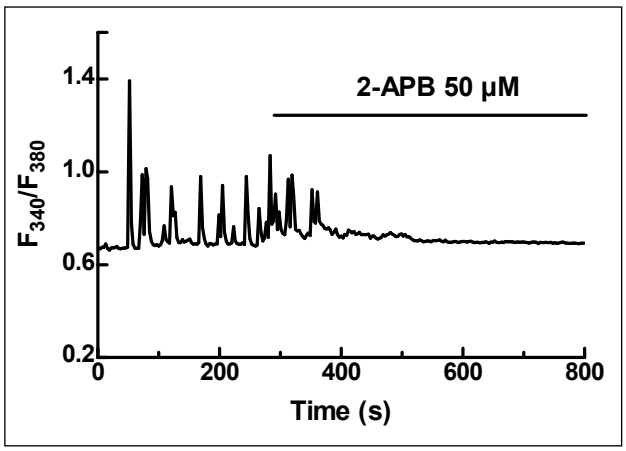

Fig. 6. IP_3R antagonist reduces Ca^{2+} oscillations. The trace represents the effect of 2-APB, a blocker of IP_3R, on spontaneously oscillating detrusor mouse cells (n=11 cells, 3 preparations). Note the inhibitory effect of the compound.

The profile of $[Ca^{2+}]_i$ oscillations in newborn mice ranged from spikes of irregular amplitude and period, frequently overlapping and occasionally grouped in "bursts" (*Fig. 1A*), to oscillations with a clear regularity both in period and shape (*Fig. 1B*). In cells with a stable pattern each oscillations was typically initiated by a slow $[Ca^{2+}]_i$ increase, and the final phase of the decay was faster than in irregular oscillations. A small percentage of cells showed isolated $[Ca^{2+}]_i$ transients of small and variable magnitude without apparent rhythmicity.

To determine the role of Ca^{2+} influx in the oscillations, we superfused detrusor cells with a Ca^{2+}-free medium. As can be observed in Figure 2, this resulted in an abrupt termination of the signal, which was restored after reintroduction of extracellular Ca^{2+}. This fast effect was not due to depletion of intracellular stores, as shown by the release of Ca^{2+} evoked by bethanechol after the suppression of oscillations in Ca^{2+} free medium *(Fig. 2B)*.

To investigate the main route of Ca^{2+} entry in oscillations we used nitrendipine, a specific blocker of voltage-operated L type Ca^{2+} channels. Superfusion with 1 µM nitrendipine inhibited the oscillations in all treated cells (8 cells, three independent experiments), with a time course similar to that induced by removal of extracellular Ca^{2+} (*Fig. 3A*). If the oscillations are mainly operated by Ca^{2+} influx through L-type channels, they would be inhibited by hyperpolarization. *Fig. 3B* shows that pinacidil, a ATP-dependent K^+ channel opener which hyperpolarizes detrusor muscle (20), suppressed or strongly reduced the spontaneous signal in 17 out of 21 cells, with an average inhibition of 53% in amplitude (0.262±0.023 before and 0.134±0.033 after pinacidil, ratio units, $P<0.001$) and 69% in frequency (0.036±0.004 before and 0.012±0.003 Hz after treatment, $P<0.001$). This effect was evident within 300-500 seconds of treatment, and further evidenced the role of voltage-operated Ca^{2+} channels.

It has been postulated that small inward currents carried by T-type Ca^{2+} channels, which are activated by slight depolarizations of plasma membrane, can trigger influx through L-type Ca^{2+} channels to induce oscillations (13, 21). *Fig. 3C* shows that application of NNC 55-0396, a specific blocker of T-type Ca^{2+} channel blocker (18), suppressed the oscillations as rapidly as nitrendipine, indicating that these channels are necessary for the spontaneous activity.

Previous reports have suggested that intracellular Ca^{2+} stores are not involved in spontaneous $[Ca^{2+}]_i$ oscillations (10). To assess this, we superfused cells with thapsigargin (TPS), a specific inhibitor of the Ca^{2+} pump of the sarcoplasmic reticulum (SERCA) (*Fig. 4A*). This compound clearly impaired or suppressed oscillations after several minutes of treatment in 74% of the oscillating cells (43 in 58 cells from 7 preparations), in many cases after a transient acceleration of oscillations associated with an increase in the resting $[Ca^{2+}]_i$ (a hallmark of TPS treatment in numerous cell types due to blockade of Ca^{2+} reuptake into the stores). In 26% of the cells (15 of 58) the transient effect persisted for the time-frame of the record (*Fig. 4A*, bottom). Our result indicates that during spontaneous oscillations Ca^{2+} is continuously pumped into sarcoplasmic reticulum.

In addition to SERCA-operated pools of the endoplasmic reticulum, cell stimulation can also release Ca^{2+} from a subset of intracellular pools operated by Ca^{2+}/H^+ exchange driven by vacuolar V-type proton pumps (22-24). To investigate its participation in the spontaneous oscillations we treated detrusor cells with folimycin, a specific inhibitor of this proton pump (17). *Fig. 4B* shows that under this treatment the oscillatory

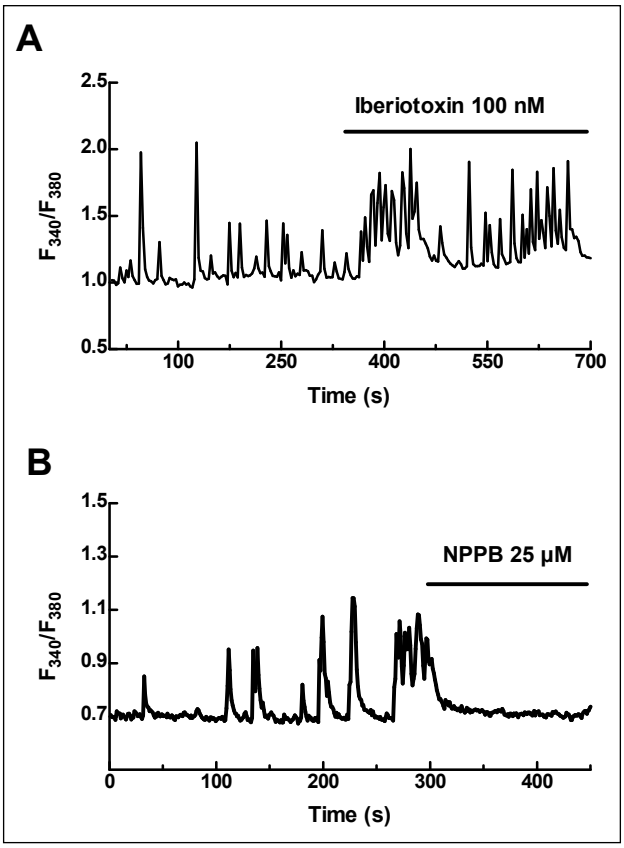

Fig. 7. Ca^{2+} oscillations of detrusor cells are modulated by Ca^{2+} -activated ion channels of sarcolemma. (**A**) The record shows two examples of the accelerating effect of iberiotoxin, a specific inhibitor of BK channels, on spontaneous $[Ca^{2+}]_i$ oscillations. Representative of 10 cells (3 preparations). (**B**) Application of NPPB, an inhibitor of Ca^{2+} -activated Cl^- channels, terminates Ca^{2+} oscillations (typical of 6 cells, 4 preparations).

signal showed a significant reduction in amplitude (0.217±0.010 and 0.130±0.024 ratio units, before and after treatment, n=36 cells, 5 preparations, $P<0.001$) and frequency (0.039±0.003 and 0.018±0.005 Hz, before and after treatment, $P<0.001$). The time course of the effect ranged from immediate to a 5 min delay, and suggested the involvement of acidic stores.

Another approach to study the role of intracellular stores is the use of inhibitors for the main Ca^{2+} releasing channels of the intracellular pools, *i.e.* IP_3 receptors (IP_3R) and ryanodine receptors (RyR). *Fig. 5A* shows the effect of 8-Br-cADPr, a selective inhibitor of RyR (25). After 300-400 second of treatment with 8-Br-cADPr spontaneous oscillations were significantly reduced both in amplitude (0.126±0.029 ratio units) and in frequency (0.018±0.005 Hz) compared to the control period (0.275±0.017 ratio units and 0.033±0.004 Hz, $P<0.001$ for both parameters, n=15). The alkaloid ryanodine slowly depletes RyR-mediated Ca^{2+} pools by locking the RyR in open conformation (26). Ryanodine reduced (in 300-500 seconds) the frequency of oscillations in 27 of 46 recorded cells (0.033±0.003 Hz before and 0.012±0.002 Hz after ryanodine, $P<0.001$, 6 preparations, *Fig. 5B*). When ryanodine was combined with a low concentration of caffeine, which releases Ca^{2+} by binding to RyR (27), the inhibition of oscillations was even stronger (total blockade in 8 of 11 cells, 4 preparations) and faster, after a transient acceleration due to the releasing effect of caffeine (*Fig. 5B*, bottom trace). Taken together, our data support a role for RyR in spontaneous oscillations in bladder smooth muscle cells.

To test the possible implication of IP_3R in spontaneous oscillations we used the antagonist 2-APB. Application of 50 µM 2-APB suppressed the oscillations after several minutes in 9 of 11 studied cells (*Fig. 6*), inducing a statistically significant decrease both in amplitude (0.214±0.029 before and 0.027±0.018 after treatment, ratio units, $P<0.001$) and frequency (0.030±0.003 before and 0.005±0.004 Hz after 2-APB, $P<0.001$, n=11).

The Ca^{2+} released from intracellular stores can modulate Ca^{2+} influx through activation of Ca^{2+}-dependent K^+ channels, which results in sarcolemma hyperpolarization and closure of voltage-operated Ca^{2+} channels (19, 28). If this were the case, inhibitors of potassium channels would be expected to increase some parameters of $[Ca^{2+}]_i$ oscillations. Accordingly, application of 100 nM iberiotoxin, a specific inhibitor of Ca^{2+}-activated BK channels, increased the frequency of the oscillations in 10 of 15 studied cells (*Fig. 7A*, 0.032±0.006 and 0.048±0.006 Hz, before and after iberiotoxin, $P<0.001$), following a variable delay of 150-600 seconds. Another possible mechanism for store-mediated Ca^{2+} influx during the oscillations is the operation of Ca^{2+} -activated Cl^- channels ($Cl^-_{Ca^{2+}}$). To assess this possibility we applied NPPB, an inhibitor of this type of channels (16). *Fig. 7B* shows that this treatment blocked the oscillatory signal within two minutes.

DISCUSSION

Our study demonstrates that urinary bladder smooth muscle from newborn mice show spontaneous $[Ca^{2+}]_i$ oscillations involving the activation of T- and L-type Ca^{2+} channels modulated by the release from intracellular Ca^{2+} stores, including acidic stores.

Previous information about spontaneous Ca^{2+} signals in urinary smooth muscle is very scarce. This study is the first direct characterization of spontaneous $[Ca^{2+}]_i$ signals in detrusor cells, although spontaneous $[Ca^{2+}]_i$ signals have been reported in strips of bladder muscle (12) and in rat whole bladders (3). A recent study briefly reports the presence of single-cell resolved spontaneous oscillations in mouse detrusor (2). In isolated pig detrusor cells a recent study using inward Cl^- currents as index of $[Ca^{2+}]_i$ signals reported NO-induced oscillations due to Ca^{2+} influx through channels insensitive to L-type Ca^{2+} channel blockers (29). The $[Ca^{2+}]_i$ oscillations described in our study are driven by L-type Ca^{2+} channels mediated Ca^{2+} entry, as revealed by the clear-cut and reversible inhibition induced by Ca^{2+} free solution and nitrendipine. That Ca^{2+} influx contributes directly to oscillations, and not by refilling of the internal stores, is shown by the fast inhibition of oscillations in Ca^{2+} free medium when the stores are still releasable (*Fig. 2B*). The key role of L-type Ca^{2+} channels mediated Ca^{2+} influx entails that control of sarcolemma potential is crucial in oscillations. In fact, the two main patterns of oscillations described in our study *(Fig. 1)* clearly resemble the two patterns of spontaneous action potentials recently described in mouse detrusor cells (2).

In contrast with our results, previous reports describe that intracellular Ca^{2+} stores are not a requisite for $[Ca^{2+}]_i$ oscillations in intact detrusor bundles (12). The difference could be due to methodology, since $[Ca^{2+}]_i$ determination in muscle strips would fail to detect non-propagating oscillations limited to single cells. We present here several evidences demonstrating that in single cells the oscillations require the presence of operative Ca^{2+} pools. First, blockade of the main channels releasing Ca^{2+} from the stores, IP_3R and RyR, dissipate the oscillations. Although it could be argued that 2-APB and caffeine could perturb Ca^{2+} homeostasis at other levels (*e.g.* gap-junctions or cAMP levels), the specific RyR antagonist 8-Br-cADPr has a clear effect. Second, inhibition of the sarcoplasmic SERCA Ca^{2+} pumps with TPS modifies the oscillatory pattern, indicating that reuptake into the stores

continuously shapes the global oscillations (by refilling the stores and/or by controlling $[Ca^{2+}]_i$ domains in the proximity of Ca^{2+} channels). A third line of evidence involving intracellular stores is the novel finding of folimycin-induced effects. Reports in smooth muscle (30) and other models (31) reveal that NAADP, the most potent Ca^{2+} releasing messenger known hitherto, releases Ca^{2+} from acidic stores operated by V-type proton pumps, in keeping with our observation that folimycin inhibits $[Ca^{2+}]_i$ oscillations.

Any theory to explain spontaneous oscillations must integrate both Ca^{2+} influx *via* L-type Ca^{2+} channels and Ca^{2+} release from internal stores, the two main mechanisms required by this signal. Stores can participate in $[Ca^{2+}]_i$ oscillations not only by reinforcing the ascending phase through release of Ca^{2+}, but also controlling the sarcolemma potential, the key factor regulating Ca^{2+} influx in excitable cells. Detrusor cells display a low sarcolemma potential (10) due to a background sodium permeability (8), so that even small depolarizations due to subtle changes in the activity of ion channels or Na^+/Ca^{2+} exchanger could trigger oscillations. At any given moment, voltage-dependent Ca^{2+} entry rate is controlled by hyperpolarizing K^+ channels and by depolarizing $Cl^-_{Ca^{2+}}$ and T-type Ca^{2+} channels, all of them present in detrusor cells(7, 28, 29). In fact Fry *et al.* have proposed that low voltage activated T-type Ca^{2+} channels, which open within a narrow "window potential" close to the resting potential of detrusor cells, could activate influx through L channels (by local depolarization) (13), also confirmed by other reports (21). This influx, in turn, would increase bulk cytosolic Ca^{2+} and at the same time would terminate the transient through activation of K^+ channels *via* CICR from the stores (13).

It is worthy to note that our results regarding the involvement of K^+ channels are not due to the side-effect of inhibitors of the Na^+/Ca^{2+} exchanger, a problem recently reported for this type of drugs (32), since we have not addressed the role of this transporter in our study.

It is a feature of several types of smooth muscle cells the presence of crosstalk between sarcolemma potential and calcium stores through modulation of Ca^{2+}-activated ion channels (19, 28, 33-36). Our results using inhibitors of $Cl^-_{Ca^{2+}}$ channels (NPPB), and both blockers (iberiotoxin) or openers (pinacidil) of Ca^{2+} -activated K^+ channels, indicate that spontaneous Ca^{2+} oscillations can be generated by the cross-talk between these channels after intracellular Ca^{2+} release. It is possible that Ca^{2+} released from internal stores sets the sarcolemma potential at values optimal for initiation of Ca^{2+} influx because the proximity of acidic stores and calcium channels in muscle cells (37). In fact, $[Ca^{2+}]_i$ signals based on repetitive release from acidic internal stores (23) are modulated by NAADP in non excitable cells (38), and in arterial myocytes acidic stores have been reported to behave as a trigger pool initiating Ca^{2+} signals (39).

Intracellular stores could also shape oscillations through removal of Ca^{2+} ions during the descending phase of each transient. In fact, a recent report in heart myocytes shows that inhibition of Ca^{2+} stores pumps reduce the rate of Ca^{2+} decays (40). The presence of vigorous Ca^{2+} removing mechanisms near sarcolemma channels can facilitate oscillations because it would relieve the desensitization or activation of Ca^{2+} -sensitive channels (such as L-type channels of calcium-activated K^+ and Cl^- channels).

It must be noted that, since the present study has been performed at room temperature (20-25°C), the percentage of cells showing oscillations at 37°C would be likely higher, both in adults and in newborn animals. Previous studies have shown that cooling from 37°C to 22°C reduces Ca^{2+} currents and Ca^{2+}-dependent K^+ currents in ureter muscle, while Ca^{2+} -dependent Cl^- channels are enhanced (41). These effects resulted in prolongation and slight amplitude increases of action potentials and $[Ca^{2+}]_i$ transients induced by imposed depolarization. Therefore, from our results we could expect faster and slightly enhanced oscillations at 37°C. In fact, this is in keeping with the effect of temperature on spontaneous action potentials in bundles of urinary bladder smooth muscle (12) and on spontaneous $[Ca^{2+}]_i$ oscillations in airway smooth muscle (42).

The origin of spontaneous activity in detrusor muscle has been attributed to ICC-like cells placed in the borders of muscle bundles (9), although other authors report interstitial cells as transmitters of spontaneous $[Ca^{2+}]_i$ signals originated in smooth muscle cells (43). The bundles develop action potentials driven by Ca^{2+} influx, which depolarize sarcolemma and feeds back further Ca^{2+} influx through L-type channels. This activity spreads within individual bundles with little propagation between adjacent ones (10). This pattern allows active bladder accommodation to the volume of urine without development of voiding (coordinated) contractions (1). The presence of spontaneous $[Ca^{2+}]_i$ oscillations in newborn individuals has a physiological relevance in view of the poor neural control of micturition at this stage of development, when muscle mechanisms are more important and the incidence of spontaneous contractions is higher compared to adult mice (4).

Acknowledgements: This study was financed by grants BFU-2007-60563 (Ministery of Education and Science, Spain) and PRI0769A (Junta de Extremadura, Spain) and RETICEF RD06/0013/1012. Francisco E. Martin-Cano has a Fellowship from Ministery of Education and Science.

Conflict of interests: None declared.

REFERENCES

1. Brading AF. Spontaneous activity of lower urinary tract smooth muscles: correlation between ion channels and tissue function. *J Physiol* 2006; 570: 13-22.
2. Meng E, Young JS, Brading AF. Spontaneous activity of mouse detrusor smooth muscle and the effects of the urothelium. *Neurourol Urodyn* 2008; 27: 79-87.
3. Kanai A, Roppolo J, IkedaY, *et al*. Origin of spontaneous activity in neonatal and adult rat bladders and its enhancement by stretch and muscarinic agonists. *Am J Physiol Renal Physiol* 2007; 292: F1065-F1072.
4. Szigeti GP, Somogyi GT, Csernoch L, Szell EA. Age-dependence of the spontaneous activity of the rat urinary bladder. *J Muscle Res Cell Motil* 2005; 26: 23-29.
5. Ganitkevich VY, Isenberg G. Contribution of Ca(2+)-induced Ca^{2+} release to the $[Ca^{2+}]$i transients in myocytes from guinea-pig urinary bladder. *J Physiol* 1992; 458: 119-137.
6. Wu C, Sui G, Fry CH. The role of the L-type Ca(2+) channel in refilling functional intracellular Ca(2+) stores in guinea-pig detrusor smooth muscle. *J Physiol* 2002; 538: 357-369.
7. Chow KY, Wu C, Sui GP, Fry CH. Role of the T-type Ca2+ current on the contractile performance of guinea pig detrusor smooth muscle. *Neurourol Urodyn* 2003; 22: 77-82.
8. Thorneloe KS, Nelson MT. Properties of a tonically active, sodium-permeable current in mouse urinary bladder smooth muscle. *Am J Physiol Cell Physiol* 2004; 286: C1246-C1257.
9. McCloskey KD, Gurney AM. Kit positive cells in the guinea pig bladder. *J Urol* 2002; 168: 832-836.
10. Hashitani H, Fukuta H, Takano H, Klemm MF, Suzuki H. Origin and propagation of spontaneous excitation in smooth muscle of the guinea-pig urinary bladder. *J Physiol* 2001; 530: 273-286.
11. Herrera GM, Heppner TJ, Nelson MT. Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am J Physiol Regul Integr Comp Physiol* 2000; 279: R60-R68.

12. Hashitani H, Brading AF. Ionic basis for the regulation of spontaneous excitation in detrusor smooth muscle cells of the guinea-pig urinary bladder. *Br J Pharmacol* 2003; 140: 159-169.
13. Fry CH, Sui G, Wu C. T-type Ca^{2+} channels in non-vascular smooth muscles. *Cell Calcium* 2006; 40: 231-239.
14. Alcon S, Camello PJ, Garcia LJ, Pozo MJ. Activation of tyrosine kinase pathway by vanadate in gallbladder smooth muscle. *Biochem Pharmacol* 2000; 59: 1077-1089.
15. Morales S, Camello PJ, Alcon S, Salido GM, Mawe G, Pozo MJ. Coactivation of capacitative calcium entry and L-type calcium channels in guinea pig gallbladder. *Am J Physiol Gastrointest Liver Physiol* 2004; 286: G1090-G1100.
16. Dai Y, Zhang JH. Role of Cl^- current in endothelin-1-induced contraction in rabbit basilar artery. *Am J Physiol Heart Circ Physiol* 2001; 281: H2159-H2167.
17. Manabe T, Yoshimori T, Henomatsu N, Tashiro Y. Inhibitors of vacuolar-type H(+)-ATPase suppresses proliferation of cultured cells. *J Cell Physiol* 1993; 157: 445-452.
18. Huang L, Keyser BM, Tagmose TM, *et al*. NNC 55-0396 [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride]: a new selective inhibitor of T-type calcium channels. *J Pharmacol Exp Ther* 2004; 309: 193-199.
19. Pozo MJ, Perez GJ, Nelson MT, Mawe GM. Ca^{2+} sparks and BK currents in gallbladder myocytes: role in CCK-induced response. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G165-G174.
20. Seki N, Karim OM, Mostwin JL. Effect of pinacidil on the membrane electrical activity of guinea pig detrusor muscle. *J Pharmacol Exp Ther* 1992; 263: 816-822.
21. Yanai Y, Hashitani H, Kubota Y, Sasaki S, Kohri K, Suzuki H. The role of Ni(2+)-sensitive T-type Ca^{2+} channels in the regulation of spontaneous excitation in detrusor smooth muscles of the guinea-pig bladder. *BJU Int* 2006; 97: 182-189.
22. Yamasaki M, Churchill GC, Galione A. Calcium signalling by nicotinic acid adenine dinucleotide phosphate (NAADP). *FEBS J* 2005; 272: 4598-4606.
23. Camello-Almaraz C, Pariente JA, Salido G, Camello PJ. Differential involvement of vacuolar H^+-ATPase in the refilling of thapsigargin- and agonist-mobilized Ca^{2+} stores. *Biochem Biophys Res Commun* 2000; 271: 311-317.
24. Camello C, Pariente JA, Salido GM, Camello PJ. Role of proton gradients and vacuola H(+)-ATPases in the refilling of intracellular calcium stores in exocrine cells. *Curr Biol* 2000; 10: 161-164.
25. Sethi JK, Empson RM, Bailey VC, Potter BV, Galione A. 7-Deaza-8-bromo-cyclic ADP-ribose, the first membrane-permeant, hydrolysis-resistant cyclic ADP-ribose antagonist. *J Biol Chem* 1997; 272: 16358-16363.
26. Laporte R, Hui A, Laher I. Pharmacological modulation of sarcoplasmic reticulum function in smooth muscle. *Pharmacol Rev* 2004; 56: 439-513.
27. Pessah IN, Stambuk RA, Casida JE. Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg2+, caffeine, and adenine nucleotides. *Mol Pharmacol* 1987; 31: 232-238.
28. Herrera GM, Nelson MT. Differential regulation of SK and BK channels by Ca^{2+} signals from Ca^{2+} channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J Physiol* 2002; 541: 483-492.
29. Kajioka S, Nakayama S, Seki N, Naito S, Brading AF. Oscillatory membrane currents paradoxically induced via NO-activated pathways in detrusor cells. *Cell Calcium* 2008; 44: 202-209.
30. Boittin FX, Galione A, Evans AM. Nicotinic acid adenine dinucleotide phosphate mediates Ca^{2+} signals and contraction in arterial smooth muscle via a two-pool mechanism. *Circ Res* 2002; 91: 1168-1175.
31. Galione A. NAADP, a new intracellular messenger that mobilizes Ca^{2+} from acidic stores. *Biochem Soc Trans* 2006; 34: 922-926.
32. Liang GH, Park S, Kim JA, Choi S, Suh SH. Stimulation of large-conductance Ca^{2+}-activated K^+ channels by the Na^+/Ca^{2+} exchanger inhibitor dichlorobenzamil in cultured human umbilical vein endothelial cells and mouse aortic smooth muscle cells. *J Physiol Pharmacol* 2008; 60: 43-50.
33. Kajioka S, Nakayama S, Asano H, Brading AF. Involvement of ryanodine receptors in muscarinic receptor-mediated membrane current oscillation in urinary bladder smooth muscle. *Am J Physiol Cell Physiol* 2005; 288: C100-C108.
34. Bolton TB, Imaizumi Y. Spontaneous transient outward currents in smooth muscle cells. *Cell Calcium* 1996; 20: 141-152.
35. Sanders KM. Invited review: mechanisms of calcium handling in smooth muscles. *J Appl Physiol* 2001; 91: 1438-1449.
36. Nelson MT, Cheng H, Rubart M, *et al*. Relaxation of arterial smooth muscle by calcium sparks. *Science* 1995; 270: 633-637.
37. Kinnear NP, Wyatt CN, Clark JH, *et al*. Lysosomes co-localize with ryanodine receptor subtype 3 to form a trigger zone for calcium signalling by NAADP in rat pulmonary arterial smooth muscle. *Cell Calcium* 2008; 44: 190-201.
38. Menteyne A, Burdakov A, Charpentier G, Petersen OH, Cancela JM. Generation of specific Ca^{2+} signals from Ca^{2+} stores and endocytosis by differential coupling to messengers. *Curr Biol* 2006; 16: 1931-1937.
39. Kinnear NP, Boittin FX, Thomas JM, Galione A, Evans AM. Lysosome-sarcoplasmic reticulum junctions. A trigger zone for calcium signaling by nicotinic acid adenine dinucleotide phosphate and endothelin-1. *J Biol Chem* 2004; 279: 54319-54326.
40. Mackiewicz U, Lewartowski B. The effect of sarcoplasmic reticulum Ca^{2+} leak on contractile activity of guinea pig heart myocytes Ca^{2+} epends on activity of sarcoplasmic reticulum Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger. *J Physiol Pharmacol* 2009; 59: 287-300.
41. Burdyga TV, Wray S. On the mechanisms whereby temperature affects excitation-contraction coupling in smooth muscle. *J Gen Physiol* 2002; 119: 93-104.
42. Featherstone NC, Jesudason EG, Connell MG, *et al*. Spontaneous propagating calcium waves underpin airway peristalsis in embryonic rat lung. *Am J Respir Cell Mol Biol* 2005; 33: 153-160.
43. Hashitani H, Yanai Y, Suzuki H. Role of interstitial cells and gap junctions in the transmission of spontaneous Ca^{2+} signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 2004; 559: 567-581.

Received: January 8, 2009
Accepted: November 6, 2009

Author's address: Dr. Pedro J Camello, Department of Physiology, Faculty of Veterinary Sciences, University of Extremadura, 10071 Caceres, Spain; Phone: +34 927257000 Extensions 51321/51290; E-mail: pcamello@unex.es